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Short communication

Determination of total tiopronin in human plasma by LC–ESI–MS using tris (2-carboxy-ethyl) phosphine as reducing reagent and methyl acrylate as derivatization reagent for the thiol group

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Abstract

A quantitative method for the determination of total tiopronin (TP) in human plasma was developed by liquid chromatography with electrospray ionisation (ESI) mass spectrometric detection. After reduction with tris (2-carboxy-ethyl) phosphine (TCEP) and derivatization with methyl acrylate (MA) for the thiol group of TP, plasma samples were processed successively by deproteinization and solid phase extraction. *N*-acetyl-L-cysteine (NAC) was selected as internal standard undergoing the same treatment as TP. The method was validated that it could meet the need of biological analysis. The lower limit of quantitation (LLOQ) of TP in plasma was 0.02 μg/mL. Finally, the method was successfully applied to a pharmacokinetic study in 20 healthy Chinese male volunteers after an oral dose of 200 mg TP tablets.

Keywords: Tiopronin; LC–ESI–MS; Methyl acrylate; Tris (2-carboxy-ethyl) phosphine; Plasma; Pharmacokinetics

1. Introduction

Tiopronin (TP) is a derivative of glycine containing active thiol group (Fig. 1A). It can be used for the therapy of hepatitis, cataract and rheumatoid arthritis [1–3]. In biological fluid, TP exists as several molecular species including free thiol (T-SH), symmetrical disulphide (T-S-S-T), mixed disulphide with another low molecular thiols (T-S-S-R) and mixed disulphide with proteins bearing free thiol group (T-S-S-P), and the different molecular species of tiopronin can interchange with each other. So the specific molecular species of TP cannot be determined so far [4]. Chemical reduction is required to split the disulphide bridge to determine total tiopronin in plasma. Furthermore, due to the absence of chromophore in the structure of TP, several papers reported the determination of tiopronin by HPLC with fluorescence detection after derivatization with *N*-substituted maleimide following reduction [5,6]. But the production of diastereoisomers increased the complexity of chromatograms. TP could also be derivatized with ThioGlo^{TM} 3 and *p*-bromophenacyl bromide (*p*-BPB) and the products were determined by HPLC–FLD [7] and HPLC–UV [8], respectively. These provided alternative methods for the determination of TP in biological samples. But UV and fluorescence detection need special reagents containing chromophore and a fine adjustment of mobile phase were commonly needed for the complete separations of TP from endogenous impurities. Mass spectrometry is a sensitive and generally applicable detection method for most compounds including those containing no chromophores. Kenji Matsuura developed a GC/MS [9] and a LC/MS/MS [10] methods for the determination of TP using acrylic acid ester as the derivatization reagent without generating asymmetric carbon. Recently, Ma et al. reported another LC–MS method for the determination of deoxidized TP using Vitamin C and 2-mercaptoethanol (2-ME) as the reducer and stabilizer. But because of the strong polarity of TP, a suitable counter ion should be added in the mobile phase [11]. In this paper, we applied Matsuura's derivative method and firstly introduced tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP) as the reducing reagent for TP, developed a new LC–MS method for the determination of total TP in human plasma with lower limit of quantitative (LLOQ) of 0.02 μg/mL for 0.2 mL plasma which was more sensitive than the previous method [11]. *N*-acetyl-L-cysteine

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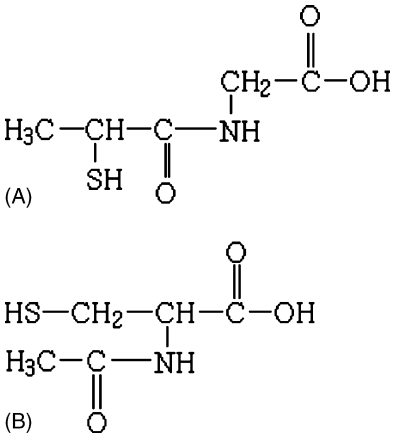

Fig. 1. The chemical structures of tiopronin (A) and *N*-acetyl-L-cysteine (B).

(NAC) was selected as internal standard (Fig. 1B). The method was successively applied to a pharmacokinetic study in 20 healthy Chinese male volunteers after an oral dose of 200 mg TP tablets.

2. Experimental

2.1. Chemicals

Tiopronin was supplied by Beijing Kawin Bio-Tech Co. Ltd. (Beijing, China); tris (2-carboxy-ethyl) phosphine hydrochloride, methyl acrylate (MA) and *N*-acetyl-L-cysteine were purchased from Sigma–Aldrich Corporation (St. Louis Missouri, USA); methanol and acetonitrile of HPLC-reagent grade were purchased from Concord Tech. Co. (Tianjin, China); all other chemicals were of analytical-reagent grade. Tiopronin tablets were supplied by Henan Xinyi pharmaceutical Co. Ltd. (Henan, China). Redistilled water, prepared from deionized water, was used throughout the study.

2.2. LC/MS conditions

The chromatographic separation was performed by an Agilent 1100 HPLC system equipped with a G1312A binary pump, a G1313A autosampler and a G1322A degasser (Agilent Corp., USA) using an Eclipse XDB-C_8 (150 mm × 4.6 mm, 5 μm) analytical column and a XDB-C_8 (12.5 mm × 4.6 mm, 5 μm) guard column (Agilent Corp., USA). The column temperature was maintained at 30 °C. The mobile phase consisted of acetonitrile–methanol–0.2% acetic acid (19:19:62, v/v/v) and was pumped at a flow rate of 0.4 mL/min.

A MSD quadrupole mass spectrometer equipped with a an electrospray ionization (ESI) source (Agilent Corp., USA) was used for the detection of analytes in the negative ion mode. Quantification was performed using selected ion monitoring (SIM) of the product ion *m/z* 161.9 for both derivatives of TP and NAC. The optimized ion source parameters were set as follows: the drying gas flow, 10 L/min; the drying gas temperature, 300 °C; the nebulizer pressure, 30 psi; the capillary voltage, 4000 V; the fragmentor voltage, 150 V. Data acquisition and instrument control were performed by A.09.01 ChemStation (Agilent Corp., USA).

2.3. Preparation of standard solutions and reactant solutions

A stock solution of TP was prepared at 1 mg/mL in water. This solution was further diluted with water to give a series of standard solutions with the concentrations of 0.2, 0.4, 0.5, 2, 4, 5, 20, 40, 50 μg/mL. Internal standard (IS) solution was prepared by dissolving 10 mg NAC in 10 mL water and diluted further with water to give a concentration of 10 μg/mL. The diluted standard and IS solutions were prepared freshly before use.

TCEP solution was prepared at 25 mg/mL in water. MA solution was prepared at 5% (v/v) in acetonitrile.

2.4. Sample preparation

To a 0.2-mL aliquot of plasma, 30 μL of IS solution and 60 μL of TCEP solution were added. The mixture was vortex-mixed for 1 min, left at room temperature for 10 min and then added 0.4 mL of 0.1 mol/L Tris–HCl buffer (pH 9.1, 5 mmol/L EDTA) and 20 μL of MA solution. The mixture was vortex-mixed for 1 min, left at room temperature for 30 min and then deproteinized with 1 mL acetone. The precipitate was removed by centrifugation at 1.2×10^3 g for 10 min and the supernatant was concentrated to less than 0.2 mL under a stream of nitrogen at 55 °C. 0.2 mL of 1 mol/L HCl solution and 0.4 mL of water were added to the residue. The solution obtained was transfered to an Oasis HLB 3 cc cartridge which was washed with 3 mL of methanol and 3 mL of water successively in advance and washed by 2 mL of 5% methanol, eluted by 2 mL of methanol. The elution was collected in a 5 mL glass tube and evaporated to dryness under a stream of nitrogen at 55 °C. The residue was dissolved in 200 μL of mobile phase and a 10-μL aliquot of the resulting solution was injected for LC–MS analysis.

2.5. Method validation

Standard samples were prepared by spiking 20 μL of the appropriate standard solutions to 0.2 mL of blank human plasma. Effective concentrations in plasma samples were 0.02, 0.05, 0.2, 0.5, 2 and 5 μg/mL for TP. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 0.04, 0.4 and 4 μg/mL, respectively. The calibration line was constructed by weighted ($1/x^2$) least-square linear regression analysis of the peak area ratio of TP–IS versus the concentration. The accuracy and precision were assessed by determining six replicated QC samples at three concentration levels on three different validation days. The accuracy was expressed by the relative error (RE) and the precision by the relative standard deviation (RSD). The lower limit of quantitation was defined as the lowest concentration of calibration samples at which an acceptable accuracy of ± 20% and a precision below 20% for 6 replicated samples could be obtained.

We investigated the stability of total TP in plasma. The three concentration levels (0.05, 0.4 and 4 μg/mL) of spiked samples

were assessed after storage at room temperature for 4 h, below −40 °C for 4 weeks and after three freeze–thaw cycles. The stability of the processed samples was investigated for 24 h at room temperature. All these samples were determined duplicately and compared to the nominal concentration.

2.6. Pharmacokinetic study of TP in human

To demonstrate the reliability of this method for the study of pharmacokinetics of TP, we applied the method to determine TP concentrations in plasma samples of 20 healthy male volunteers after an oral administration of 200 mg TP. Experimental protocols were approved by the Medicinal Ethical Committee of Bethune International Peace Hospital. Twenty healthy male volunteers have a mean age of 23.2 years (range 19–28 years) and a mean weight of 62.3 kg (range 52–70 kg). On the basis of medical history inquiry, clinical examination and laboratory investigation (haematology, blood biochemistry and urine analysis), all subjects met the inclusion criteria and they all gave written informed consent. After an overnight fasting, subjects were given an oral dose of 200 mg TP tablets with 200 mL of water. Standard lunch and dinner were given 5 h and 10 h after administration, respectively. Two millilitre of blood samples were drawn from ulnar vein into heparinized tubes before (0 h) and 1, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 24, 36, 48, 72, 96 h after administration. The blood samples were centrifuged at 1.2×10^3 *g* for 10 min. Plasma samples were separated and stored at −40 °C till analysis.

Pharmacokinetic parameters were calculated by non-compartmental method. The maximum TP concentration (C_{max}) and the corresponding peak time (T_{max}) were observed from the individual drug plasma concentration–time profile. The elimination rate constant (k_e) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration–time profile. The elimination half-life ($T_{1/2}$) was calculated as $0.693/k_e$. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity was calculated as $AUC_{0-t} + C_t/k_e$, where C_t is the last measurable concentration.

3. Results and discussion

3.1. Reduction, derivatization and purification of TP in plasma sample

TP contains a thiol group, which is easily oxidized to disulfides with endogenous thiols [4]. To determine exactly the concentration of TP in plasma, reduction of thiol group is necessary. Several reducing reagents for thiol compound most commonly used are 2-mercaptoethanol (2-ME), dithiothreitol (DTT), sodium borohydride ($NaBH_4$), tributylphosphine (TBP). They all have some shortcomings. 2-ME and DTT contain thiols, they will consume some reactant during the reaction; $NaBH_4$ needs high concentration or long time for complete reaction and TBP is amyctic and explosive. While tris (2-carboxy-ethyl) phosphine hydrochloride is colorless, tasteless and easily soluble in water. Furthermore, it can react with thiol in short time at ambient temperature [12]. In this study, 60 μL of TCEP (25 μg/mL) reacted with 0.2 mL plasma samples containing 5 μg/mL TP completely and quickly.

During the early stage of method development, we planed to determine deoxidized TP released from plasma by TCEP, and simplify the preparation of samples without the step of the derivatization with MA. But because TP and IS have strong polarity, they are not appropriate to be extracted by liquid–liquid extraction (LLE) or solid phase extraction (SPE) method. Protein precipitation was selected to prepare the plasma samples. But TP could not be separated from the endogenous purities due to weak retention on the bonded silica phase. After TP and IS were reacted with MA, the derivatives had relatively decreased polarity, which retained better on XDB C_8 column and could be separated completely. Fourthermore, derivatization of thiol group can protect it from oxidation. So, after reduction, derivatization was also taken for theTP before determination.

Methyl acrylate was used as the derivatization reagent of TP according to the reports of Kenji Matsuura [9,10]. But we used a less amount of MA in this paper (5% × 20 μL) than that in the previous method (7% × 100 μL) [10] for the same amount of plasma sample. We used the calibration samples at the highest concentration (5 μg/mL) to demonstrate the amount of MA used in this paper was sufficient for the derivatization of TP because a more amount could not increase the response of the derivative.

For the purification of derivatives in plasma samples, SPE obtained higher recoveries and less impurity than LLE.

3.2. Optimization of LC–MS condition

TP and IS have the same molecular weight and both of them can react with MA to produce derivatives also having the same molecular weight. But the derivatives could be separated completely under the HPLC conditions mentioned above. At first, we selected *m/z* 248.1, the quasi-molecular ion of the derivatives, as the monitoring ion at lower fragment voltage (50 V), but endogenous impurities from plasma interfere with TP–MA. Later, we found TP–MA and IS–MA could easily lost the fragment of MA to give product ion *m/z* 161.9 which corresponded to quasi-molecular ions of both TP and IS ($[M–H]^-$) and the abundance of that ion increased when fragment voltage was increased. When we increased fragment voltage to 150 V and selected ion *m/z* 161.9 as the monitoring ion, the interference from the blank plasma was decreased. So we selected ion *m/z* 161.9 for quantitation of TP. The chromatograms of blank plasma, blank plasma spiked with TP and IS and a volunteer's sample spiked with IS are shown in Fig. 2. The retention time was 6.0 min for IS and 6.9 min for TP, respectively.

3.3. Method validation

A good linearity between the peak area ratio of TP to IS (R) and the concentration of TP (C) was obtained over the plasma concentration range of 0.02–5 μg/mL. A regression equation was obtained as follows: $R = (3.093 \pm 0.017)\ C + (0.0144 \pm 0.0012)$, the correlation coefficient was 0.9991 ± 0.0002 ($n = 6$).

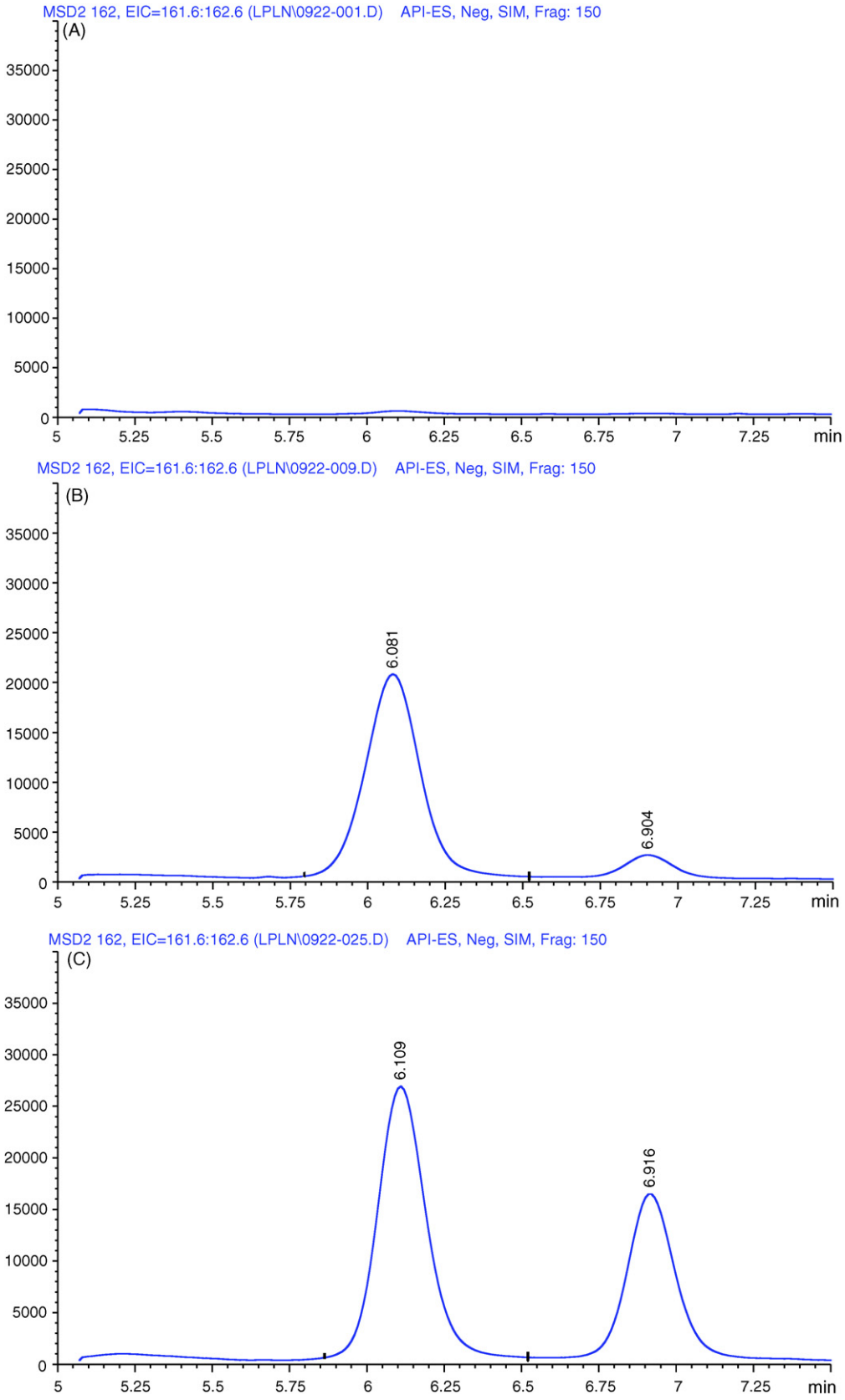

Fig. 2. Typical chromatograms (SIM) of TP–MA and NAC–MA in plasma samples. (A) blank plasma, (B) blank plasma spiked with TP (0.04 μg/mL) and IS, (C) a human plasma sample 10 h after administration of 200 mg TP tablets. The SIM ion was *m/z* 161.9 for TP–MA and NAC–MA.

Table 1
Accuracy and precision for the determination of TP in plasma ($n = 18$)

Added C (μg/mL)	Found C (μg/mL)	Intra-day, RSD (%)	Inter-day, RSD (%)	RE (%)
0.04	0.042	3.9	9.7	5.6
0.40	0.390	2.1	12.5	−1.6
4.00	3.900	2.4	11.8	−3.5

Table 2
Stability of plasma samples under indicated conditions

Nominal C (μg/mL)	Ambient temperature, 4 h		Three freeze–thaw cycles		−40 °C, 4 weeks	
	Found (μg/mL)	RE (%)	Found (μg/mL)	RE (%)	Found (μg/mL)	RE (%)
0.05	0.052	4.0	0.056	12.2	0.042	−15.9
0.40	0.394	−1.5	0.445	11.2	0.363	−9.2
4.00	3.571	−10.7	3.694	−7.6	4.093	2.3

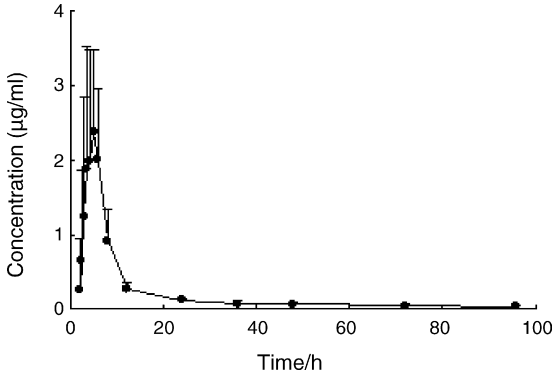

Fig. 3. Mean plasma concentration–time curve of TP in 20 healthy volunteers after an oral dose of 200 mg TP tablet.

The results of the precision and accuracy of the method are summarized in Table 1. The intra-day and inter-day RSDs of TP were less than 3.9% and 12.5%, respectively. The REs of the method were −3.5–5.6%. The LLOQ was 0.02 μg/mL.

The results of stability of plasma samples are summarized in Table 2. It is shown that plasma samples are stable at room temperature for 4 h, after three freeze–thaw cycles and below −40 °C for 4 weeks. The processed samples are stable at room temperature for at least 24 h.

3.4. Pharmacokinetic study of TP in Chinese volunteers

The mean concentration–time curve in 20 Chinese volunteers after an oral administration of 200 mg TP is shown in Fig. 3. Similar to the results reported by Carlsson [13] and Hercelin [14], a slow absorption and a slow elimination were observed after oral administration of TP. The main pharmacokinetic parameters were calculated. C_{max} was 2.42–4.85 μg/mL, T_{max} was 3.5–6.0 h, $T_{1/2}$ was 15.9–56.5 h, $AUC_{0–96\,h}$ was 12.0–25.27 μg h/mL and $AUC_{0–\infty}$ was 12.79–29.72 μg h/mL.

4. Conclusion

A sensitive and reproducible LC–MS method for the determination of total TP in human plasma has been developed and validated using TCEP as the reducing reagent and MA as the derivatization reagent for the thiol group. The process of sample preparation was simple and timesaving. The method has been successfully applied to a pharmacokinetic study of TP in human and could detect the plasma concentration 96 h after administration of 200 mg TP. The pharmacokinetic results corresponded to those obtained by other HPLC methods.

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